IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:

MESSADEK, Jallal

Application Ser. No. 09/945,391

Filed: August 31, 2001

GLYCINE BETAINE AND ITS USE

Group Art Unit: 1614

Examiner: Clinton T. Ostrup

DECLARATION OF JALLAL MESSADEK PURSUANT TO 37 C.F.R. 1.132

1. I, Jallal Messadek, a citizen of Belgium and residing in Liege, Belgium, make the following declaration in support of the above-referenced patent application filed and now pending in the United States Patent and Trademark Office. I am over the age of eighteen, under no disability, and fluent in English and understand the following statements made in support of this application.

2. In support of the response to the Office Action in the underlying International Application PCT/BE00/00021, tests have been performed under my supervision showing the difference existing between glycine betaine and gamma butyrobetaine. These tests are disclosed and more fully set forth in the following paragraphs and attachments which are fully incorporated by reference herein.

3. For these tests, the following material was used:

Gamma butyrobetaine from SIGMA-ALDRICH®;

Anhydrous betaine BETAFIN® (Finnsugar Bioproducts, Cultor, Helsinki, Finland);

Rats (Wistar), males, weight between 250 and 300 grams, n=5/lot and per test;

Sodium Thiopentol;

Aggregometer CHRONOLOG COULTRONIC S.A. France;

ADP from Laboratoires Stago France;

Automated Coagulation timer, Medtronic Hemotec Inc., Englewood, Colorado, USA;

4. The METHODS used in the tests are as follows:

A. Platelet aggregation induced ex-vivo

The aggregation tests were made in accordance to the methods Cardinal & Flower. Pharmacol. Method. 1980. and to American Journal of Clinical Pathology, 1989; 92: 676-679. Sureney. JD. Whole Blood aggregometry.

After a keeping period of 8 days, the rats are subjected to a fasting for 12 hours. Betaine is subcutaneous injected one hour before blood sampling. The rats are then anaesthetized with sodium Thiopental administered at a dose of 200 mg/Kg and the blood samples are taken by intracardiac puncture on a trisodium citrate solution (1 volume of solution at 3, 8 % citrate for 9 volumes of blood).

The platelet aggregation is then realized by adding an agonist (ADP).

B. Activated coagulation time (kaolin)

This test explores the intrinsic coagulation pathway. One hour or 24 hours after subcutaneous administration of the betaine, 0.8 ml total blood by intracardiac way is taken in a container HR, HemoTec. These tubes contain the kaolin activator. (Method HemoTec., automated coagulation timer manufactured by MEDTRONIC HEMOTEC Inc., Englewood, CO., USA).

C. Principle of laser-induced thrombosis

(Seiffge D. et al., 1989; Weichter W. et al., 1983)

In this model, lesion of the vascular wall is induced by a laser beam. This beam causes a limited

lesion of the vascular endothelium (only 1 to 2 cells are destroyed). This laying base of the sub-endothelium, which is a thrombogenetic surface, results in the adherence of platelets via glycoprotein Ib. This adherence of platelets is followed by the activation thereof. They form pseudopods and secrete the content of their granules. This activation results in the appearance of glycoproteins IIb-IIIa which are necessary for the aggregation of the platelets between them. This lesion is induced in the mesenteric microcirculation of the rat. It is immediately followed by the formation of a thrombus (in a few seconds). This thrombus, which rapidly enlarges under the influence of the flow of blood, embolises before being formed again.

5. Performed Tests

For said study, Whitstar rats, male, have been used. Their weight is comprised between 200 and 250 grams. After an 8-day stabilization period, the rats were subjected to fasting for 12 hours.

Glycine betaine and gamma butyrobetaine were administered by subcutaneous way one hour and 24 hours before the tests. Rats were then anaesthetized using Thiopental sodium 200 mg/kg and the mesentery was opened for samplings and laser experiments.

6. **Test 1: Activated coagulation time (Kaolin) at different doses**

A. Anticoagulation activity at 200 mg/kg

	Activated coagulation time (Seconds)	Variation of activated coagulation time (%)
Control NaCl 0.9%	53.8 +/- 4.96	0
Gamma butyrobetaine 200 mg/kg	209.2 +/-15.94	+288%
Glycine betaine 200 mg/kg	481.6 +/- 37.78	+ 795%

In order to be in the same experimental conditions, the doses used correspond to the doses (200

mg/kg) mentioned in WO 97/06795). It has been observed a high mortality for the rats to which gamma butyrobetaine is administered (2 rats out of 5), the remaining living rats having respiration problems and a very opaque blood. Further rats have been used for completing the group of rats treated with gamma butyrobetaine. The rats of the betaine group, according to the experimentator" staying approximately normal".

It has then been decided to continue the tests with lower doses as mentioned in Applicant's specification and more conform to therapeutic dose. The dose of 200 mg/kg by direct injection of the example 4 in WO 97/06795 corresponds to an injection of 14 grams of butyrobetaine for a human of 70kg.

B. Anticoagulation activity at 50 mg/kg

	Activated coagulation time (seconds)	Variation of activated coagulation time (%)
Control NaCl 0.9%	53.8 +/- 4.96	0
Gamma butyrobetaine 50 mg/kg	88.6+/- 9.68	+65%
Glycine betaine 50 mg/kg	199.6 +/- 15.97	+ 271%

C. Anticoagulant activity at 10 mg/kg

	Activated coagulation time (Seconds)	Variation of activated coagulation time (%)
Control NaCl 0.9%	53.8 +/- 4.96	0
Gamma butyrobetaine 10 mg/kg	66.2 +/-4.96	+23%
Glycine betaine 10 mg/kg	125.4 +/- 14.79	+ 133%

D. **Comments:** Glycine betaine has an anticoagulant activity largely greater than the

activity of gamma butyrobetaine. When comparing examples I, 1 and I, 2, it appears that glycine betaine administered at 50 mg/kg has the same performance than butyrobetaine administered at a dose of 200 mg/kg (dose causing the death of some rats). It means that more than 4 times gamma butyrobetaine is required for having substantially the same anticoagulation activity.

7. **Test II: comparison of the efficiency of glycine betaine versus gamma butyrobetaine 24 hours after the subcutaneous administration of 10 mg/kg.**

A. Parameters of the thrombosis induced by laser 24 hours after administration of the products

	Number of shots	Number of emboli	Embolisation time (Minutes)
Control NaCl 0.9%	2.4 +/- 0.54	5.2 +/- 0.83	2.90 +/- 1.81
Gamma butyrobetaine 10 mg/kg	2.4 +/- 0.54	3 +/- 0.7	2.2 +/-0.44
Glycine betaine 10 mg/kg	3.6 +/- 0.54	1 +/- 0.7	0.0 +/- 0.0

B. Activated coagulation time (kaolin) 24 hours after administration of the products

	Activated coagulation time (Seconds)	Variation of activated coagulation time (%) with respect to the control
Control NaCl 0.9%	53.8 +/- 4.96	0
Gamma butyrobetaine 10 mg/kg	54.62 +/- 6.34	+1.5%
Glycine betaine 10 mg/kg	87.6 +/- 10.19	+ 63%

After 24 hours, the gamma butyrobetaine has no more any anticoagulation activity, while glycine betaine remains active.

C. Platelet aggregation induced ex vivo with ADP 24 hours after administration of the products

	Amplitude (Ohms)	Velocity (Ohms/minutes)
Control NaCl 0.9%	13 +/- 1	9 +/- 1
Gamma butyrobetaine 10 mg/kg	11.2 +/- 1.78	7.6 +/- 0.54
Glycine betaine 10 mg/kg	5.2 +/- 1.3	3.8 +/- 0.83

	Amplitude variation with respect to the control (%)	Velocity variation with respect to the control (%)
Gamma butyrobetaine 10 mg/kg	-15	-16
Glycine betaine 10 mg/kg	-60	-58

D. **Comments**

Glycine betaine remains efficient 24 hours after administration, while gamma butyrobetaine has substantially no more activity. This difference exists for all the examined parameters.

It has to be observed that the anticoagulation activity of glycine betaine at 10 mg/kg 24 hours after administration is substantially equal to the activity of gamma butyrobetaine at 50 mg/kg one hour after administration (Test II, 2 and I, 2).

8. **Conclusion**

A. Glycine betaine has an anticoagulation activity, an anti-thrombotic activity and an anti aggregation activity which are quite higher than these for gamma butyrobetaine, and this for

all the studied doses. The activity of betaine is also observable on a longer time. With respect to the dose mentioned in WO 97/06795 (200 mg/kg of gamma butyrobetaine), a four time lower dose (50 mg/kg) of glycine betaine is sufficient for obtaining equivalent results. This higher activity is unexpected.

B. The two molecules, despite having similarities in their structures, have however major differences in theirs formula, molecular weights, melting points and toxicities. These differences could explain the higher pharmacological effects in vivo of the glycine betaine with respect to gamma butyrobetaine.

	Formula	Molecular weight	Melting point (C)
Gamma Butyrobetaine	$C_7 H_{16} NO_2$	181.66	220
Glycine Betaine	$C_5 H_{11} NO_2$	117.15	303

C. Glycine betaine has not only a better activity, but also has a lesser toxicity than gamma butyro-betaine, an advantage which is quite decisive for the preparation of a medicine for treating humans.

D. Indeed, the LD 50 (lethal dose) for glycine betaine is:

10800 mg/kg in case of oral administration, and 830 mg/kg in case of parenteral administration,

E. While the LD 50 for gamma butyrobetaine is:

3500 mg/kg in case of oral administration, and 475 mg/kg in case of parenteral administration.

F. Glycine betaine can thus in no way be considered as being an equivalent of gamma butyrobetaine.

G. I attach also to said affidavit a digest of tests made by eminent Professors, said tests being:

- Induced focal cerebral ischemia on rats under the supervision of Professor Christoph de HAEN, Milan - Italy

- Free radical induced femoral artery thrombosis and bleeding tests on hamster by Professor Marc HOYLAERTS, Centre for Molecular and Vascular Biology, University of Leuven, Belgium
- Human blood Flow perfusion experiments on collagen and on endothelial cells by Professor Giuseppe REMUZZI from Mario Negri Cardiovascular Institute, Bergamo - Italy
- Thrombin generation assay experiments on human blood by Professor HC. HEMKER & Professor Suzette BEGUIN both of Cardiovascular Institute of Maastricht – The Netherlands

9. I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: October 21th 2003
(21/10/2003)

Jallal Messadek